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SUPERNUMERARY LIMB INDUCTION IN *AMBYSTOMA* LARVAE BY FROG TISSUE IMPLANTS¹

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ABSTRACT. Pieces of kidney and/or lung from adult frog (*Rana pipiens*) when implanted under the dorsal skin of the lower arm (mid-radius/ulna level) of *Ambystoma maculatum* larvae, induced supernumerary limbs in over 75% of the cases. Liver was completely ineffective in inducing ability. However, liver did not lower the inducing capacity of lung in combination grafts. When the insertion hole was distal to the graft, the percentage of induction by kidney was slightly higher than when the insertion hole was proximal. In each case, the supernumerary limb developed from the site of the insertion hole. The original epidermis covering the insertion injury site very likely became the wound epidermis for the supernumerary regenerate. In comparison to frog kidney, newt (*Notophthalmus viridescens*) kidney was a poor inducer. Limbs with *Ambystoma* cartilage grafts or limbs simply injured without tissue implants did not exhibit supernumerary limb induction.

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INTRODUCTION

It has long been known that an injury is needed to initiate limb regeneration in urodele amphibians (Thornton 1968, Carlson 1974). Induction of supernumerary growths from virtually any position along the limb of newts and *Ambystoma* larvae shows the injury need not be amputation (Carlson 1974). In order for super-

numerary limbs to develop, the conditions for regeneration must be met, as they are after typical amputation. The mesodermal tissues of the limb must be damaged locally by the injury, a wound epidermis must be present over the injured tissues, and sufficient nerve fibers must grow into the area of injury (Carlson 1974, Tank and Holder 1981).

In order for regeneration to occur, a considerable number of cell divisions must occur to provide a blastema of sufficient

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size from which the missing limb parts or the supernumerary structure can be constructed. The importance of cell division for epimorphic regeneration gained considerable emphasis when Tassava and Mescher (1975) hypothesized independent cell cycle (cell division) roles for injury, nerves, and wound epidermis. According to this hypothesis, injury promotes dedifferentiation of mesodermal stump cells. The resulting mesenchymal-like cells require the presence of nerves and wound epidermis for their successful divisions (proliferation).

The supernumerary induction system provided a possible model to examine the interaction of injury, nerves, and wound epidermis in initiating the regeneration process. Earlier studies had shown implants of frog kidney and lung would induce supernumerary limbs in adult newt (*Notophthalmus viridescens*) limbs (Carlson 1971). Since the kidney tissue can be frozen and even lyophilized without adversely affecting the inductive capacity, a chemical component of the tissue may be responsible for the effect (Stevens et al. 1965, Carlson and Morgan 1967). However, the adult newt system is slow to respond. Results often were tabulated up to 70 days after the initial tissue implantation (Stevens et al. 1965). In the present study, *Ambystoma maculatum* larvae provided a rapid and consistent system with which to examine supernumerary limb induction by tissue implants.

The objectives of this study were: to determine the frequency of supernumerary limb induction in *Ambystoma* larvae by implants of different tissues from frog, newt, and *Ambystoma* sources; to determine whether the usual lack of inductions by liver implants was due to the presence of an inhibitor or to lack of an inductive stimulus, and to compare the frequency of supernumerary limb inductions by frog kidney when the implant was made via distal versus proximal insertion wounds.

METHODS AND MATERIALS

Embryos of *Ambystoma maculatum* were collected from ponds in southern Ohio and raised to hatching

age in the laboratory in aerated tap water at $22 \pm 1^\circ\text{C}$. Hatched larvae were fed live brine shrimp (*Artemia*) 3 times weekly until larvae reached a snout-tail tip length of 35–40 mm. Larvae were anesthetized with MS222 (ethyl-m-amino benzoate methane sulfonate). With warchmaker's forceps, small holes were made through the skin of the dorsal surface of the lower forelimb either over the distal 25% of the radius/ulna (distal insertion hole) or the proximal 25% of the radius/ulna (proximal insertion hole). Then a tunnel was formed under the skin in the proximal direction or distal direction, respectively. Control limbs were injured by making the tunnel but not given tissue implants. Tissues for implantation were dissected fresh, placed in ice-cold Holtfreter's solution, and used within 1 hr of dissection. Tissues for implantation included *Ambystoma mexicanum* phalangeal cartilage, adult newt kidney, and frog (*Rana pipiens* from Wisconsin) kidney, liver, and lung. Implants were cut into pieces approximately 33% the diameter of the limb and pushed into the tunnel under the skin (subcutaneously). The tissue caused the skin to protrude slightly dorsally (as a small bump) a short distance proximal or distal to the insertion wound site. Limbs were examined at 10–40 magnification daily through 3 weeks for the presence of implant tissues and the position and frequency of supernumeraries. Because the skin of small *Ambystoma* larvae is transparent, it was possible to ascertain when the implant was lost; these few cases (5%) were excluded from the sample.

Supernumerary limbs which exhibited one or more spike-like structures (digits) were classed as good inductions. Supernumerary limbs which showed an outgrowth, but without any digit-like (spike) structure, were classed as poor inductions. Limbs without outgrowth were classed as no inductions.

RESULTS AND DISCUSSION

A typical supernumerary limb resulting from the implantation of frog kidney (fig. 1) has 3 digits and was therefore classed as a "good induction." It developed from a proximal insertion site. Morphological indication of a developing supernumerary regenerate was usually apparent at 6 days and sometimes at 5 days post-implantation.

In every case of induction (table 1), the supernumerary limb grew from the site of the wound at the insertion site. Perhaps this is not surprising in that a wound epidermis was made readily available by the initial skin incision and because of the recognized importance of the wound epidermis for regeneration (Singer and Salpeter

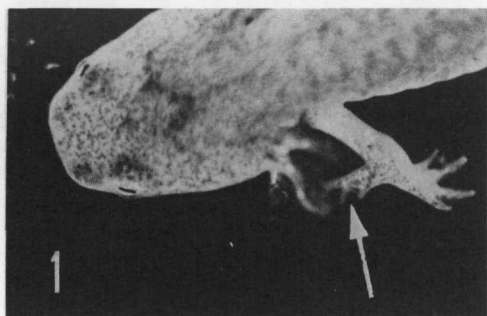


FIGURE 1. A photograph of a supernumerary limb (arrow) induced on the left limb of an *Ambystoma* larva by frog kidney implanted into a proximal wound site. This 3-digit supernumerary limb maintained its 90° angle to the host limb. The photograph was taken 14 days after implantation. 5.6×

1961, Mescher 1976). Ruben and Frothingham (1959) implanted frog kidney into *A. opacum* limbs and observed a considerably lower frequency of accessory growths from proximal wound sites (3 of 13) than from distal wound sites (11 of 13). As in the present study, distal site supernumerary regenerates grew "distad" whereas proximal site supernumerary regenerates initially grew in the proximal direction and then turned distad to become perpendicular to the host limb axis. Rubin and Frothingham (1959) also noted the insertion wound probably provided a dermis-free epithelium which developed

subsequently into a wound epidermis. Supernumeraries developing from proximal insertion wounds grew out at nearly a 90° angle to the host limb (fig. 1) whereas outgrowths from distal insertion wounds developed more parallel to the host limb, generally at an angle of 45° or less. This likely is due to the known directional influences of the wound epidermis on blastemal outgrowth (Thornton 1968) and is worthy of additional investigation.

The wound epidermis at the wound site was not sufficient by itself for supernumerary induction. Only when frog lung or frog kidney were implanted near the wound site did supernumeraries result. Liver, even in the presence of wound epidermis, was not an effective inducer. This is an important observation which argues against the suggestion of Stevens et al. (1965) that liver is an ineffective inducer in adult newt limbs because wound epidermis fails to form above the implant. A potential wound epidermis was present at the site of injury in the *Ambystoma* limbs of the present study. However, it is possible this epidermis only developed into a functional wound epidermis when kidney or lung were implanted.

Observations of successful supernumerary inductions with kidney/liver combinations suggest that liver does not produce an active inhibitor of supernumerary limb

TABLE 1
Number of supernumerary limbs induced in forelimbs of *Ambystoma* larvae by tissue implants.

Tissue Implant	Cases	Wound Site	No Induction	Poor Induction	Good Induction
Injury without implant	14	Distal	14	0	0
	8	Proximal	8	0	0
<i>Ambystoma</i> cartilage	6	Distal	6	0	0
Frog kidney	13	Distal	1	2	10
Frog kidney	15	Proximal	5	2	8
Newt kidney	5	Distal	4	0	1
Frog liver	7	Distal	7	0	0
Frog liver	9	Proximal	8	1	0
Frog lung	11	Distal	2	1	8
Frog liver/lung combinations*	9	Distal	2	2	5

*Equal amounts of liver and lung were used. The total amount of implant tissue in these combination grafts was approximately 1.5 times the quantity of single implants.

induction. The high percentages of inductions with kidney and lung (table 1) are consistent with the view expressed by Carlson and Morgan (1967) that these tissues, but not liver, provide a chemical stimulus which results in the development of a supernumerary appendage.

The fact that many of the supernumerary limbs were incomplete legs, i.e. with even single phalanges, is of potential importance with regard to the possible application of one or more of the various pattern formation models to explain accessory limb regeneration (Tank and Holder 1981).

It is clear the trauma of injury alone was insufficient to elicit outgrowth. Limbs injured without tissue implants or with *Ambystoma* cartilage or newt kidney implants either failed to show or seldom showed supernumerary limbs. Carlson and Morgan (1967) reached this conclusion earlier from results of their experiments in supernumerary induction in adult newts. Since all of the limbs in the present study were injured and had a potential wound epidermis, at least initially, then perhaps one determining factor for whether or not a supernumerary limb develops is whether or not sufficient nerve fibers grow into the tissue implant area. Alternatively frog kidney and lung may enhance the injury effect of the initial wound to provide more dedifferentiated cells (Tassava and Mescher 1975) or stimulate the epidermis at the implant site to result in functional wound epidermis (Tassava and Olsen 1982).

This system provides the opportunity to examine the chemical characteristics of frog kidney and lung essential to supernumerary induction and, by examination of the cellular events occurring or not occurring after kidney and lung versus liver implants, may provide further clues

as to why injury, nerves, and wound epidermis are so essential to epimorphic regeneration.

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